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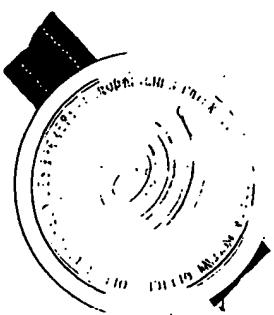
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Den Haag, den
The Hague,
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Der Präsident des Europäischen Patentamts
Im Auftrag
For the President of the European Patent Office
le Président de l'Office européen des brevets
p.o.

R. Turner
R.D.H. TURNER



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Page 2 de l'attribution**



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Expression of polysaccharide degrading enzymes in plants

FIELD OF THE INVENTION

The present invention relates to the development of
5 transgenic plants having a modified carbohydrate composition.

INTRODUCTION

It has long been an objective of the agricultural industry to develop crops having a modified carbohydrate composition, thus providing plants or plant organs more suitable for certain applications. Such modified crops provide plant products having a modified flavor, a higher content of desired saccharides or a more desirable texture. These crops may be either consumed directly or used in further processing.

In several plant species such as corn (Shannon & Garwood, 1984), pea (Bhattacharyya et al, 1990), potato (Hovenkamp-Hermelink et al, 1987), *Arabidopsis* (Caspar et al, 1985; Lin et al, 1988a; Lin et al, 1988b) and tobacco (Hanson et al, 1988), mutants with an altered carbohydrate composition have been found. This phenomenon may be attributable to mutations found mainly in enzymes involved in the synthesis of starch. Some of these mutants are already used in the food industry, such as sweet corn (Shannon & Garwood, supra), which may be directly consumed.

Mutants altered in starch metabolism may be obtained via classical techniques such as random screening procedures or cross-breeding. However, these are laborious and time consuming processes. Moreover, certain mutations may give rise to the phenotype that is screened for, but may lose other desired characteristics, or even obtain highly undesired characteristics (such as potatos having a high alkaloid content). Changing plant characteristics through genetic engineering is a precise and predictable method; the nature of the gene which is spliced into the genome is known and no undesired genes are integrated simultaneously. Finally, modification of a specific characteristic, for instance alteration of the level or nature of certain products in the mutant is often difficult or even impossible

using classical techniques. As such, genetic modification techniques will open up new strategies and lead to new products that cannot be obtained by classical techniques.

It would be clearly advantageous to develop sophisticated and predictable methods for obtaining plants having a modified carbohydrate composition, based on genetical engineering techniques.

In US patent 4,801,540, DNA fragments are disclosed encoding an enzyme capable of hydrolyzing poly (1,4- α -D galacturonide) glycan into galacturonic acid. Expression constructs are provided in which the structural gene encoding this enzyme is linked to modified regulatory regions in order to modulate the expression of the enzyme. The purpose of the invention disclosed in the application mentioned above is to decrease expression levels of the PG enzyme in order to inhibit degradation of polygalacturonic acid to control fruit ripening.

In PCT application WO 89/12386 plants and methods are disclosed in which the carbohydrate content is modified through expression in said plants of enzymes such as sucrase and levan sucrase. The object of the invention disclosed in said application is to increase the concentration of high molecular weight carbohydrate polymers in fruit in order to alter soluble solids and viscosity.

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SUMMARY OF THE INVENTION

The present invention provides transgenic plants or plant organs which have a modified polysaccharide composition and methods for the production of these plants. This is achieved via the introduction into the plant of an expression construct containing a DNA sequence encoding an enzyme which is capable of degrading plant polysaccharides.

Furthermore, depending on the products desired in planta, additional expression constructs may be introduced into the plant which contain DNA sequences encoding other enzymes which convert the degradation products resulting from the first enzymatic reaction to the desired oligo- or monosaccharides.

The DNA expression constructs provided by the present

invention for the transformation of plants are under the control of regulatory sequences which are capable of directing the expression of the selected polysaccharide modification enzymes. These regulatory sequences may also 5 include sequences capable of directing the expression of the chosen enzymes at a desired developmental stage of the plant or plant organ and/or tissue specificity.

The transgenic plants provided by the present invention find applications as new products with a modified taste 10 and/or more desirable texture, both for direct consumption and for processing.

BRIEF DESCRIPTION OF THE FIGURES

15 Figure 1. Binary vector pMOG23.
Figure 2. Genomic sequence of the alpha-amylase gene of Bacillus licheniformis as present in the vector pPROM54.
Figure 3. Synthetic oligonucleotide duplexes used for the various constructions.
20 Figure 4. Binary plasmid pMOG228. Binary vector pMOG23 containing the genomic DNA sequence encoding mature alpha-amylase from Bacillus licheniformis preceded by a methionine translation initiation codon.
Figure 5. Binary plasmid pMOG437. Binary vector pMOG23
25 containing DNA sequences encoding mature α -amylase from Bacillus licheniformis and mature glucoamylase from Aspergillus niger, both preceded by a methionine translation initiation codon and both under the control of a class-I patatin promoter from potato.

30

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides transgenic plants or plant organs which have a modified polysaccharide composition and 35 overcomes the disadvantages encountered in classical plant breeding techniques by stably introducing into the plants, DNA sequences encoding certain enzymes which are capable of polysaccharide degradation.

It was unexpectedly found that the transformation of

tobacco with a bacterial α -amylase gene (lacking a secretory signal sequence) resulted in the accumulation of maltodextrins such as maltose and maltotriose, which is indicative of α -amylase activity. This finding demonstrates 5 that it is possible to modify polysaccharide composition in planta by the introduction of a polysaccharide degrading enzyme.

The observed degradation of starch by the α -amylase enzyme is very surprising since in plant cells, starch is present in 10 specific organelles (chloroplasts, amyloplasts and the like) whereas the expressed α -amylase is expected to be present in the cytoplasm since no sequences were present to direct the α -amylase to these organelles.

Starch degrading enzymes are present in the cytoplasm of 15 plant leaf cells. However, their function in the cytoplasm has never been conclusively explained and has never been correlated to the degradation of starch, because of the compartmental division of the two entities (Caspar et al., 1989; Lin et al., 1988; Okita et al., 1979).

20

Enzymes of interest, which may be expressed in plants according to the present invention, include any enzymes or combination of enzymes which are capable of degrading plant 25 polysaccharides and those capable of further modifying the polysaccharide degradation products to the desired saccharide subunits.

Such enzymes include those which are of non-plant origin. Also intended are enzymes, originating from plants, which are expressed via recombinant DNA techniques, if necessary after 30 modification of the coding or regulatory sequences to achieve cytoplasmic or organellar expression, tissue specificity or expression at a desired plant (organ) or plant (organ) maturity stage.

Such enzymes include A) starch degrading enzymes such as 35 1) α -amylases (EC 3.2.1.1), 2) exo-1,4- α -D glucanases such as amyloglucosidase (=glucoamylase) (EC 3.2.1.3), β -amylase (EC 3.2.1.2), α -glucosidases (EC 3.2.1.20), and other exo-amylases, and 3) starch debranching enzymes, such as isoamylase (EC 3.2.1.68), pullulanase (EC 3.2.1.41), β -

amylases, and the like, B) cellulases such as exo-1,4- β -cellobiohydrolase (EC 3.2.1.91), endo-1,4- β -D-glucanase (EC 3.2.1.4), β -glucosidase (EC 3.2.1.21) and the like, C) endo-1,3/1,4- β -glucanases, D) L-arabinases, such as endo-1,5- α -L-arabinase (EC 3.2.1.99), α -arabinosidases (EC 3.2.1.55) and the like, E) galactanases such as endo-1,4- β -D-galactanase (EC 3.2.1.89), endo-1,3- β -D-galactanase (EC 3.2.1.90), α -galactosidase (EC 3.2.1.22), β -galactosidase (EC 3.2.1.23) and the like, F) mannanases, such as endo-1,4- β -D-mannanase (EC 3.2.1.78), β -mannosidase (EC 3.2.1.25), α -mannosidase (EC 3.2.1.24) and the like, G) xylanases, such as endo-1,4- β -xylanase (EC 3.2.1.8), β -D-xylosidase (EC 3.2.1.37), 1,3- β -D-xylanase, and the like, and other enzymes such as α -L-fucosidase (EC 3.2.1.51), α -L-rhamnosidase (EC 3.2.1.40), levanase (EC 3.2.1.65), inulanase (EC 3.2.1.7), and the like.

After a first polysaccharide degradation step, other enzymes may be used to modify the obtained (oligo)saccharides further. As an illustration, enzymes of particular interest to further degrade maltose, maltotriose and α -dextrans obtained with the first degradation of starch are *inter alia* maltases, α -dextrinase, α -1,6-glucosidases, and the like, resulting in the formation of glucose. If desired additional enzymes are expressed in the same plant, which are capable of modifying monosaccharides. Such enzymes include but are not limited to glucose isomerase, invertase, and the like. The source from which DNA sequences encoding these enzymes may be obtained is not relevant, provided the enzyme itself is active in the intracellular environment. The choice of the enzyme of interest may depend on the substrate specificity and/or the desired saccharide end-product.

The enzymes of interest may be produced constitutively in the transgenic plants during all stages of development. Depending on the use of the plant or plant organs, the enzymes may be expressed in a stage-specific manner, for instance during tuber formation or fruit development. Also, depending on the use, the enzymes may be expressed tissue-specifically, for instance in plant organs such as fruit, tubers, leaves or seeds.

Plant polysaccharides, as defined within the context of the present invention are intended to consist of polyhydroxy aldehydes or ketones, consisting of more than six covalently-linked monosaccharides, which are normally found in plants 5 prior to the action of the enzyme or enzymes of interest according to the present invention. Such polysaccharides are typically polymers of D-arabinose, D-fructose, D- and L-galactose, D-glucose, and D-xylose and mannose.

Saccharide subunits, the desired end-products of the 10 present invention, are defined saccharides having a shorter chain length than the original polysaccharide, including monosaccharides, which are obtained via the action of one or more enzymes of interest on the plant polysaccharides.

Transgenic plants, as defined in the context of the 15 present invention include plants (as well as parts and cells of said plants) and their progeny which have been genetically modified using recombinant DNA techniques to cause or enhance production of at least one enzyme of interest in the desired plant or plant organ.

20 Within the context of the present invention, plants to be selected include, but are not limited to crops producing edible flowers such as cauliflower (Brassica oleracea), artichoke (Cynara scolymus), fruits such as apple (Malus, e.g. domesticus), banana (Musa, e.g. acuminata), berries 25 (such as the currant, Ribes, e.g. rubrum), cherries (such as the sweet cherry, Prunus, e.g. avium), cucumber (Cucumis, e.g. sativus), grape (Vitis, e.g. vinifera), lemon (Citrus limon), melon (Cucumis melo), nuts (such as the walnut, Juglans, e.g. regia; peanut, Arachis hypogeae), orange 30 (Citrus, e.g. maxima), peach (Prunus, e.g. persica), pear (Pyrus, e.g. communis), pepper (Solanum, e.g. capsicum), plum (Prunus, e.g. domestica), strawberry (Fragaria, e.g. moschata), tomato (Lycopersicon, e.g. esculentum), leafs, such as alfalfa (Medicago, e.g. sativa), cabbages (such as 35 Brassica oleracea), endive (Cichoreum, e.g. endivia), leek (Allium, e.g. porrum), lettuce (Lactuca, e.g. sativa), spinach (Spinacia e.g. oleraceae), tobacco (Nicotiana, e.g. tabacum), roots, such as arrowroot (Maranta, e.g. arundinacea), beet (Beta, e.g. vulgaris), carrot (Daucus,

e.g. carota), cassava (Manihot, e.g. esculenta), turnip (Brassica, e.g. rapa), radish (Raphanus, e.g. sativus), yam (Dioscorea, e.g. esculenta), sweet potato (Ipomoea batatas) and seeds, such as bean (Phaseolus, e.g. vulgaris), pea 5 (Pisum, e.g. sativum), soybean (Glycin, e.g. max), wheat (Triticum, e.g. aestivum), barley (Hordeum, e.g. vulgare), corn (Zea, e.g. mays), rice (Oryza, e.g. sativa), tubers, such as kohlrabi (Brassica, e.g. oleraceae), potato (Solanum, e.g. tuberosum), and the like.

10 The choice of the plant species is determined by the intended use of the plant or parts thereof and the amenability of the plant species to transformation.

Several techniques are available for the introduction of the expression construct containing recombinant genes into 15 the target plants. Such techniques include but are not limited to transformation of protoplasts using the calcium/polyethylene glycol method (Krens *et al.*, 1982; Negrutiu *et al.*, 1987), electroporation (Shillito *et al.*, 1985; Fromm *et al.*, 1985) and microinjection (Crossway *et al.*, 1986) 20 or (coated) particle bombardment (Klein *et al.*, 1987). In addition to these so-called direct DNA transformation methods, also transformation systems involving vectors are widely available, such as viral vectors (e.g. from the cauliflower mosaic virus (CaMV), Fraley *et al.*, 1986) and 25 bacterial vectors (e.g. from the genus Agrobacterium). After selection and/or screening, the protoplasts, cells or plant parts that have been transformed can be regenerated into whole plants, using methods known in the art (Horsch *et al.*, 1985). The choice of the transformation and/or regeneration 30 techniques is not critical for this invention.

In a preferred embodiment of the present invention, the principle of the binary vector system is used (Hoekema *et al.*, 1983; European patent application EP-A-0 120 516, filing date 21.1.84) in which Agrobacterium strains are used which 35 contain a plasmid with the virulence genes and a compatible plasmid containing the gene construct to be transferred. This vector can replicate in both E. coli and in Agrobacterium, and is derived from the binary vector Bin19 (Bevan, 1984). The binary vectors as used in this example contain between

the left- and right-border sequences from the T-DNA, a NPTII-gene coding for kanamycin resistance (Bevan, 1984) and a multiple cloning site to clone in the required gene-constructs.

5 Expression of these recombinant genes involves such details as transcription of the gene by plant polymerases, translation of mRNA, etc. that are known to persons skilled in the art of recombinant DNA techniques. Only details relevant for the proper understanding of this invention are
10 discussed below.

Regulatory sequences which are known or are found to cause expression of the enzymes can be used in the present invention. The choice of the regulatory sequences used depends on the target crop and/or target organ of interest.
15 Such regulatory sequences may be obtained from plants or plant viruses, or may be chemically synthesized. Such regulatory sequences are promoters active in directing transcription in plants, either constitutively or stage and/or tissue specific, depending on the use of the plant or
20 parts thereof. These promoters include, but are not limited to promoters showing constitutive expression, such as the 35S promoter of Cauliflower Mosaic Virus (CaMV) (Guilley et al., 1982), those for leaf-specific expression, such as the promoter of the ribulose bisphosphate carboxylase small
25 subunit gene (Coruzzi et al., 1984), those for root-specific expression, such as the promoter from the glutamine synthase gene (Tingey et al., 1987), those for seed-specific expression, such as the cruciferin A promoter from Brassica napus (Ryan et al., 1989), those for tuber-specific
30 expression, such as the class-I patatin promoter from potato (Rocha-Sosa et al., 1989; Wenzler et al., 1989) or those for fruit-specific expression, such as the polygalacturonase (PG) promoter from tomato (Bird et al., 1988).

Other regulatory sequences such as terminator sequences
35 and polyadenylation signals include any such sequence functioning as such in plants, the choice of which is within the level of the skilled artisan. An example of such sequences is the 3' flanking region of the nopaline synthase (nos) gene of Agrobacterium tumefaciens (Bevan, 1984).

The regulatory sequences may also include enhancer sequences, such as found in the 35S promoter of CaMV, and mRNA stabilizing sequences such as the leader sequence of Alfalfa Mosaic Virus (AlMV) RNA4 (Brederode et al., 1980) or 5 any other sequences functioning in a like manner.

The enzymes of interest should be expressed intracellularly, in order to have access to the substrate. The DNA constructs should thus be designed in such a manner that intracellular expression is achieved.

- 10 To obtain expression in the cytoplasm of the cell, the expressed enzyme should not contain a secretory signal peptide or any other target sequence. For expression in chloroplasts and mitochondria the expressed enzyme should contain a specific so-called transit peptide for import into
- 15 these organelles. Targeting sequences that can be attached to the enzyme of interest in order to achieve this are known (Smeekens et al., 1990; van den Broeck et al., 1985; Schreier et al., 1985). If the activity of the enzyme is desired in the vacuoles a secretory signal peptide has to be present, as
- 20 well as a specific targeting sequence that directs the enzyme to these vacuoles (Tague et al., 1988). The same is true for the protein bodies in seeds. The DNA sequence encoding the enzyme of interest should be modified in such a way that the enzyme can exert its action at the correct place in the cell,
- 25 i.e. at the site where it has access to its substrate..

All parts of the relevant DNA constructs (promoters, regulatory-, stabilizing-, targeting- or termination sequences) of the present invention may be modified, if 30 desired, to affect their control characteristics using methods known to those skilled in the art.

A number of methods may be used to obtain transgenic plants in which more than one enzyme of interest is expressed. These include but are not limited to:

- 35 a. Cross-fertilization of transgenic plants each expressing a different enzyme of interest.
- b. Plant transformation with a DNA fragment or plasmid that contains multiple genes, each encoding an enzyme of interest, each containing its own necessary regulatory

sequences.

c. Plant transformation with different DNA fragments or plasmids simultaneously, each containing a gene for an enzyme of interest, using the necessary regulatory sequences.

5 d. Successive transformations of plants, each time using a DNA fragment or plasmid encoding a different enzyme of interest under the control of the necessary regulatory sequences.

10 e. A combination of the methods mentioned above.

The choice of the above methods is not critical with respect to the objective of this invention.

In one embodiment of the present invention, an α -amylase is expressed intracellularly in tobacco plants, resulting in the degradation of starch in these plants to lower molecular weight saccharides. A genomic DNA fragment encoding mature α -amylase from Bacillus licheniformis, i.e. encoding the α -amylase without the signal peptide sequence, is placed under 15 the control of the CaMV 35S promoter and enhancer sequences. The mRNA stabilizing leader sequence of RNA4 from AlMV is included, as well as the terminator and polyadenylation signal sequences of the nopaline synthase (nos) gene of Agrobacterium tumefaciens. The construct is thereafter 20 subcloned into a binary vector such as pMOG23 (deposited at the Centraal Bureau voor Schimmelcultures, Baarn, the Netherlands on January 29, 1990 under accession number CBS 25 102.90). This vector is introduced into Agrobacterium tumefaciens which contains a disarmed Ti plasmid. Bacterial 30 cells containing this construct are co-cultivated with tissues from tobacco plants, and transformed plant cells are selected on nutrient media containing antibiotics and induced to regenerate into differentiated plants on such media. The resulting plants contain the stably-integrated gene and 35 express the α -amylase intracellularly.

The α -amylase enzyme activity of the transgenic plants may be tested with direct enzyme assays using colorimetric techniques or gel assays. The assay of choice is not critical to the present invention. The protein is detectable on

Western blots with antibodies raised against α -amylase from Bacillus licheniformis.

The plants may be assayed for starch content either qualitatively by staining for starch with iodine or 5 quantitatively by NMR. Plants may be assayed for the presence of insoluble and soluble starch degradation products by using techniques as NMR and HPLC, respectively. Other methods may also be used. The choice of the method is not critical to the present invention.

10 In another preferred embodiment, both an α -amylase and a glucoamylase are expressed in potatoes. The enzymes are expressed only in the tubers of the plants. The result is the degradation of starch in tubers by both enzymes to lower molecular weight saccharides. A genomic DNA fragment encoding 15 mature α -amylase from Bacillus licheniformis and a cDNA fragment encoding mature glucoamylase from Aspergillus niger are each placed under the control of the tuber-specific promoter from a class-I patatin gene from potato. Both constructs also include the terminator and polyadenylation 20 signal sequences of the nopaline synthase (nos) gene of Agrobacterium tumefaciens. Both constructs are thereafter subcloned together into the binary vector pMOG23. This vector is introduced into Agrobacterium tumefaciens, which contains a disarmed Ti plasmid. Bacterial cells containing this 25 construct are cocultivated with tissues from potato plants and transformed plant cells are selected on nutrient media containing antibiotics, and induced to regenerate into differentiated plants on such media. The resulting plants contain the stably integrated genes. Both α -amylase and 30 glucoamylase are expressed only in the tubers of the transformed potatoes. Both enzymes are expressed intracellularly.

The α -amylase and glucoamylase enzyme activities in the transgenic tubers can be tested with various assays. The 35 assay of choice is not relevant to the present invention.

The transgenic potato tubers may be assayed for their carbohydrate composition by using techniques for the detection of soluble sugars such as HPLC and techniques for the detection of insoluble sugars such as NMR. Other methods

may also be used. The choice of the method is not critical to the present invention.

It should be understood that likewise other polysaccharide degrading enzymes can be introduced into and intracellularly 5 expressed in a plant of choice. If a second enzyme is used in addition said polysaccharide degrading enzyme, this second enzyme must be capable of using the polysaccharide degradation products generated by the action of the polysaccharide degrading enzyme. The action of this second 10 enzyme of interest effects the further degradation or conversion of these starch degradation products to the desired saccharide subunits.

The extent in which the taste and/or texture of the plants is modified can be regulated using a spectrum of measures. 15 These measures include, but are not limited to the regulation of expression of the enzyme(s) of interest with respect to expression level and spatial and/or developmental regulation of expression. Obviously, the stronger the promoter, and the better the choice of the promoter with respect to the desired 20 time and location of expression of the enzyme(s) of interest, the higher the levels of the enzyme(s) of interest will be in the plant part of choice. Consequently, also the effect of the action of the enzyme(s) will be the highest if the choice of the enzyme(s) is optimal. When different enzymes 25 are expressed in a plant, the ratios of the respective enzymes can be chosen in order to obtain the desired effect (e.g. the desired sweetness). Alternatively, the location within the cell (the cellular compartment or organelle) may be chosen in such a way that the desired mode of expression 30 is obtained. Combinations of the above measures can also be used to obtain the desired effect. Such methods to influence the mode and level of expression are well within the limits of the artisan.

Some variation in expression levels is sometimes observed 35 as a result of varying copy number and/or site of integration of the transforming DNA. This natural variation can be used to select the desired individual plants from the pool of transgenic plants that have the desired characteristics in terms of sweetness tecture and the like. These individual

plants can be used for multiplication and/or cross-breeding with other varieties.

Transgenic plants or plant organs (such as flowers, fruits, leaves, roots, tubers) having a higher content of polysaccharide degradation products and consequently a modified flavor and/or a desired texture, may be used as a new product either as such or in processed form. Examples of such uses are the production of baby foods, juices, sauces, pastes, concentrates, sweeteners, jams, jellies, syrups, and animal feeds. Grains having an altered carbohydrate composition may be used in the productions of baked products, for example, which have a modified taste. Tobaccos having an altered carbohydrate composition have a modified taste and aroma.

15 Alternatively, the polysaccharide degradation products may be extracted from the plant or plant organs and used as such, for instance as a sweetener, or in various processes.

The following examples are provided as a means of illustrating the instant invention, and are not intended to 20 limit the invention.

Example 1

Construction of the binary vector pMOG23.

In this example the construction of the binary vector 25 pMOG23 (deposited at the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands, on January 29, 1990, under accession number CBS 102.90) is described.

The binary vector pMOG23 (Fig. 1) is a derivative of vector Bin19 (Bevan, 1984). To obtain pMOG23, the vector 30 Bin19 is changed in a way not essential to the present invention, using techniques familiar to those skilled in the art of molecular biology. Firstly, the positions of the left border (LB) and the right border (RB) are switched with reference to the neomycin phosphotransferase gene II (NPTII) 35 gene. Secondly, the orientation of the NPTII gene is reversed giving transcription in the direction of LB. Finally, the polylinker of Bin19 is replaced by a polylinker with the following restriction enzyme recognition sites: EcoRI, KpnI, SmaI, BamHI, XbaI, SacI, XhoI, and HindIII.

Example 2Cloning of the α -amylase gene of *Bacillus licheniformis*

In this example the α -amylase gene from *Bacillus licheniformis* is tailored and cloned in an expression cassette. Any other gene may be cloned in a similar way as is described here for the α -amylase gene.

All transformations in this example are done in *E. coli* strain DH5 α .

10

a. Tailoring of the α -amylase gene of *Bacillus licheniformis*.

The α -amylase gene (Fig. 2) from *Bacillus licheniformis* is present in the *Bacillus* vector pPROM54, which is described in European Patent Application 224294. The plasmid pPROM54 has been deposited at the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands on November 5, 1985, under accession number CBS 696.85.

The plasmid is digested with XbaI and BclI. The XbaI/BclI fragment is cloned in plasmid pUC18 digested with XbaI and BamHI, resulting in plasmid pMOG318. A SalI/BamHI fragment is synthesized with pMOG318 as a template with PCR technology, creating the BamHI site by use of a mismatch primer (the position of the created BamHI site is indicated in Fig. 2). The SalI/BamHI PCR fragment is cloned in plasmid PIC-19R (Marsh et al., 1984) digested with SalI and BamHI, resulting in plasmid pMOG319. The SalI fragment from pMOG318 (the second SalI site is present in pUC18), that contains the 5' end of the α -amylase gene, is cloned in pMOG319 digested with SalI. This results in plasmid pMOG320 that contains the entire α -amylase gene.

b. Construction of vector pMOG18.

The expression cassette of pROK1 (Baulcombe et al., 1986) is cloned as an EcoRI/HindIII fragment into pUC18. This cassette contains the Cauliflower Mosaic Virus (CaMV) 35S promoter on an EcoRI/BamHI fragment and the nopaline synthase (nos) transcription terminator of *Agrobacterium tumefaciens* on a BamHI/HindIII fragment. The promoter fragment consists of the sequence from -800 to +1 (both inclusive) of the CaMV

promoter. Position +1 is the transcription initiation site (Guilley *et al.*, 1982). The sequence upstream of the NcoI site at position -512 is deleted and this site is transformed into an EcoRI Site. This is done by cutting the expression 5 cassette present in pUC18 with NcoI, filling in the single-stranded ends with Klenow polymerase and ligation of an EcoRI linker.

The resulting plasmid is cut with EcoRI, resulting in the deletion of the EcoRI fragment carrying the sequences of the 10 35S promoter upstream of the original NcoI site. The BamHI/HindIII fragment, containing the nos terminator is replaced by a synthetic DNA fragment (Oligonucleotide duplex A, Fig. 3) containing the leader sequence of RNA4 of Alfalfa Mosaic Virus (AlMV) (Brederode *et al.*, 1980). This is done by 15 cleavage with BamHI, followed by cleavage with HindIII and ligation of the synthetic DNA fragment. The BamHI site and three upstream nucleotides are deleted by site-directed mutagenesis.

In the resulting plasmid, the BamHI/HindIII fragment 20 containing the nos terminator is reintroduced. The gene encoding beta-glucuronidase (originating from plasmid pRAJ 275; Jefferson 1987) was ligated in as an NcoI/BamHI fragment, resulting in plasmid pMOG14.

From the literature it is known that duplication of the 25 sequence between -343 and -90 increases the activity of the 35S promoter (Kay *et al.*, 1987). To obtain a promoter fragment with a double, so-called enhancer sequence, the enhancer fragment from plasmid pMOG14 is isolated as an AccI/EcoRI-fragment and subsequently blunt-ended with Klenow 30 polymerase. The thus-obtained fragment is introduced in pMOG14 cut with EcoRI and blunt-ended, in such a way that the border between the blunt-ended EcoRI and AccI sites generates a new EcoRI site. The resulting plasmid pMOG18 contains the 35S CaMV promoter with a double enhancer sequence, the leader sequence of RNA4 from AlMV and the nos terminator in an expression cassette still present as an EcoRI/HindIII fragment.

c. Cloning of the α -amylase gene from *Bacillus licheniformis*

in the binary vector.

Plasmid pMOG320 is digested with HgaI and BamHI. The HgaI/BamHI fragment is cloned together with the synthetic oligonucleotide duplex B (Fig. 3) into pMOG18 digested with 5 NcoI and BamHI, resulting in plasmid pMOG322. The β -glucuronidase gene is thus replaced by the coding sequence for the mature α -amylase of Bacillus licheniformis preceded by the ATG triplet encoding the methionine translation initiation codon. Plasmid pMOG18 contains the 35S promoter 10 and enhancer of Cauliflower mosaic virus (CaMV), the nopalitin synthase (nos) terminator from Agrobacterium tumefaciens and the RNA4 leader sequence of Alfalfa mosaic virus (AlMV). The resulting construct does not contain coding information for a signal peptide. The entire construct is spliced out with 15 EcoRI and HindIII and transferred into the binary vector pMOG23 digested with EcoRI and HindIII. The resulting plasmid has been designated pMOG228 (Fig.4).

The chimeric α -amylase gene on the binary plasmid pMOG228 is mobilized, in a triparental mating with the E.coli K-12 20 strain DH5 α (pRK2013) (Ditta et al., 1980), into Agrobacterium strain LBA4404, that contains a plasmid with the virulence genes necessary for T-DNA transfer to the plant (Hoekema et al., 1983).

25 Example 3

Transformation of tobacco.

Tobacco (Nicotiana tabacum cv. SR 1) is transformed by co-cultivation of plant leaf disks (Horsch et al., 1985) with Agrobacterium tumefaciens, containing the binary vector 30 pMOG228 with the α -amylase gene. Transgenic plants are selected on kanamycin resistance. The transgenic plants are assayed for activity of the enzyme of interest. Expressors are analyzed more thoroughly and used in further experiments.

35 Leaf discs of about 5 x 5 mm are cut from leaves of axenically grown plants of Nicotiana tabacum SR1. The discs are floated for 20 minutes in MS-medium (Murashige & Skoog, 1962) containing 30 g/L sucrose with 1% (v/v) of a culture of

Agrobacterium tumefaciens LBA4404(pMOG228) (10^9 cells/ml). Subsequently, the discs are briefly dried on filter paper and transferred to plates containing solid medium consisting of MS-medium, containing 30 g/L sucrose, 7 g/L agar, 1 mg/L 5 kinetin and 0.03 mg/L naphthyl acetic acid (NAA). Two days later the discs are transferred to plates containing the same medium plus 500 mg/L carbenicillin. One week after that, the discs are again transferred to plates containing the same medium, this time with about 50 mg/L kanamycin to select for 10 transgenic shoots. Discs are transferred to fresh plates with three week intervals. Developing shoots are excised and transferred to pots containing solid medium consisting of MS-medium, containing 30 g/L sucrose, 100 mg/L kanamycin and 100 mg/L cefotaxime for root development. After roots have 15 developed the plants are transferred to the soil. The plants are tested for expression of the gene of interest.

Example 4

Alpha-amylase expression in transgenic tobacco plants

20 Alpha-amylase activity is determined by the method described by Saito (1973) at 56 °C. Units are defined in this case as the amount of enzyme giving a reduction of the absorbance at 690 nm by 10% in 10 minutes. Specific activity for the Bacillus licheniformis α -amylase is 8.7×10^5 U/mg 25 protein. The tip of one of the top leafs (about 100 mg) is cut off and homogenized in 100 μ l α -amylase assay buffer (Saito, 1973). The homogenate is spun down for 10 minutes in an Eppendorf centrifuge. The supernatant is collected and assayed for protein and α -amylase content. Control-plants had 30 levels of activity at or below the detection limit.

In the 62 transgenic plants obtained the measured expression levels, as determined by the method of Saito (1973) vary between 0 and 3.29 U/ μ g protein. Based on the specific activity of the enzyme, these levels correspond to 0 35 = 0.38 % of the total amount of soluble protein. The average is 0.11 % of the total amount of soluble protein. The protein was clearly present intracellularly, since no significant amount of α -amylase activity is detected in the extracellular fluid that is isolated by vacuum infiltration of the leaves

with buffer, followed by collection of the fluid by centrifugation (Sijmons et al., 1990). These results were confirmed with immunological detection of the Bacillus licheniformis α -amylase on Western blots, also showing that 5 the protein is indeed the desired α -amylase. Further confirmation was obtained by running extracts and extracellular fluid on polyacrylamide-SDS gels. After electrophoresis, the gels were incubated in 0.04 M Tris/HCl pH 7.4 for 3 hours with 6 changes of buffer to renature the 10 enzymes. The gels were overlayed with 0.25% potato Lintner starch, 0.75% agar in 0.05 M Tris/HCl pH 7.4 containing 1 mM CaCl₂, incubated overnight at 37 °C and subsequently stained with 1mM I₂/0.5 M KI in water. Alpha-amylase activity is detected as a clear zone in the overlay (Lacks & Springhorn, 15 1980). In the transgenic plants an α -amylase is detected with the same molecular weight of 50.000 as that of the Bacillus licheniformis α -amylase.

Tobacco plants expressing α -amylase are pale light green (chlorotic) and somewhat retarded in growth as compared to 20 control plants.

Example 5

Carbohydrate analysis of transgenic tobacco plants

Qualitatively the starch content in transgenic tobacco 25 leaves, collected at the end of the photoperiod, is determined by destaining the leaves overnight under shaking in 96% ethanol, followed by staining for starch with 5.7 mM I₂ and 43.3 mM KI in 0.2 N HCl. Leaves containing starch stain black-blue, while leaves lacking starch stain brownish- 30 yellow (Caspar et al., 1985).

Approximately 2.5 g portions of leaf material (stored in deep-freeze) obtained from control and transformed (good α -amylase expressors) plants were homogenized in 10 ml water at 4 °C with an ultra-turrax. Microscopic inspection revealed 35 that no intact cells remained. After removal of the cell fragments by centrifugation, the glucose oligomer content in the green-colored supernatant was determined. The filtered samples were analyzed via HPLC on an Aminex HPX-42A column (300 mm x 7.8 mm, 85 °C) using water as the eluent. The

presence of maltose and maltotriose were detected in the samples of the transformed plants and not in the control (untransformed) plants. The results are shown in the table, below.

5

TABLE

Saccharides extracted from tobacco leaves and analyzed on an Aminex HPX-42A-HPLC column

	<u>Preparation</u>	<u>Saccharide</u>	<u>mg Saccharide/g wet material</u>
10	Control	Maltotriose	undetectable
		Maltose	undetectable
15	Transgenic	Maltotriose	0.34
		Maltose	1.73

20

Example 6

Cloning of a cDNA encoding mature glycoamylase - from Aspergillus niger

25

A. Isolation of poly A⁺ RNA from Aspergillus niger

About 1.10⁸ spores of Aspergillus niger strain DS 2975 (deposited at the Centraal Bureau voor Schimmelcultures on 30 august 10, 1988, under number CBS 513.88) are inoculated in 100 ml pre-culture medium containing (per liter): 1 g KH₂PO₄; 30 g maltose; 5 g yeast-extract; 10 g casein-hydrolysate; 0.5 g MgSO₄.7H₂O and 3 g Tween 80. The pH is adjusted to 5.5. After growing overnight at 34°C in a rotary shaker, 1 ml of 35 the growing culture is inoculated in a 100 ml main-culture containing (per liter): 2 g KH₂PO₄; 70 g malto-dextrin (Maldex MDO₃, Amylum); 12.5 g yeast-extract; 25 g casein-hydrolysate; 2 g K₂SO₄; 0.5 g MgSO₄.7H₂O; 0.03 g ZnCl₂; 0.02 g CaCl₂; 0.05 g MnSO₄.4 H₂O and FeSO₄. The pH is adjusted to 40 5.6. The mycelium is grown for 140 hours and harvested. 0.5 g of dry mycelium is frozen with liquid nitrogen and ground. Subsequently the material is homogenized with an Ultra turrax (full speed, 1 minute) at 0°C in 10 ml 3-M LiCl, 6 M Urea and

maintained overnight at 4°C as described by Auffray and Rougeon (Eur. J. Biochem. 107, 303-314 (1980). Total cellular RNA is obtained after centrifugation at 16,000 g and solved in 3 ml 10 mM Tris-HCl (pH 7.4), 0.5% SDS and two times extracted with phenol:chloroform:isoamylalcohol (50:48:2).
5 The RNA is precipitated with ethanol and redissolved in 1 ml 10 mM Tris-HCl (pH 7.4), 0.5% SDS. For poly A⁺ selection the total RNA sample was heated for 5 minutes at 65°C, adjusted to 0.5 M NaCl and subsequently applied to an oligo(dT)-
10 cellulose column. After several washes with a solution containing 10 mM Tris pH 7.0, 0.5% SDS and 0.1 mM NaCl, the poly A⁺ RNA is collected by elution with 10 mM Tris pH 7.0 and 0.5% SDS.

15 B. Preparation and cloning of a cDNA encoding glucoamylase

For the synthesis of the first strand of the cDNA 5 µg of poly A⁺ RNA, isolated according to example 6A, is dissolved in 16.5 µl H₂O and the following components are
20 added: 2.5 µl RNasin (30 U/µl), 10 µl of a buffer containing 50 mM Tris, 6 mM MgCl₂ and 40 mM KCl, 2 µl 1 M KCl, 5 µl 0.1 M DTT, 0.5 µl oligo(dT)₁₂₋₁₈ (2.5 mg/ml), 5 µl 8 mM dNTP-mix, 5 µl BSA (1 mg/ml) and 2.5 µl Moloney MLV reverse transcriptase (200 U/µl). The mixture is incubated for 30 minutes at 37°C
25 and the reaction is stopped by adding 10 µl 0.2 M EDTA and 50 µl H₂O. An extraction is performed using 110 µl chloroform and after centrifugation for 5 minutes the waterlayer is collected and 110 µl 5 M NH₄Ac and 440 µl absolute ethanol (-20°C) is added. Precipitation is done in a dry ice/ethanol
30 solution for 30 minutes. After centrifugation (10-minutes at 0°C) the cDNA/mRNA pellet is washed with 70% ice-cold ethanol. The pellet is dried and dissolved in 20 µl of H₂O.

Isolation of a cDNA encoding glucoamylase is performed with
35 the Polymerase Chain Reaction. Two oligonucleotides are synthesized, based on the nucleotide sequence of glucoamylase. G1 cDNA published by Boel *et al.* (EMBO J. 3, 1097-1102, 1984).

Oligo 1: 5' CCCTACGAATT CAGATCTTCACCGCCAGGTGTCAGTCAC 3'
 Oligo 2: 5' GGGTACGGGCCCTCATGAATGTGATTCCAAGCGCGCG 3'

With these two oligonucleotides the region encoding the mature enzyme preceded by an ATG codon is amplified.

The polymerase chain reaction is performed according to the supplier of the Taq-polymerase (Cetus) using 1.5 µl of the solution containing the reaction product of the first strand synthesis and 0.5 µg of each of the oligonucleotides.

10 Amplification is performed in a DNA amplifier of Perkin Elmer/Cetus. After 25 cycles of 2 minutes at 94°C, 2 minutes at 55°C and 3 minutes at 72°C the reaction mixture is deproteinized by subsequent phenol and chloroform extractions. The DNA is precipitated, redissolved in a buffer

15 containing 10 mM Tris pH 7 and 0.1 mM EDTA and subsequently digested with EcoRI and Sma I. The digested DNA is ligated to pTZ18R, also completely digested with EcoRI and SmaI, and the ligation mixture is transformed to E. coli. Several transformants are grown in shake flasks and plasmid DNA is isolated.

20 The plasmid DNA's are digested with several restriction enzymes and 5 preparations with the expected restriction pattern are identified. Determination of the nucleotide sequence reveals that two plasmids contain a complete cDNA encoding mature glucoamylase G1, comparable to the one

25 isolated by Boel et al. (see above).

Example 7

30 Cloning of both α-amylase from *Bacillus licheniformis* and glucoamylase from *Aspergillus niger* in potato

In this example a DNA fragment encoding mature α-amylase from *Bacillus licheniformis* and a cDNA encoding a glucoamylase from *Aspergillus niger* are cloned in a tuber-specific expression cassette. All transformations in this example are done in *E. coli* strain DH5α.

A. Construction of the expression cassette

For the construction of the expression cassette for tuber-specific expression, the promoter from a class-I patatin gene of potato (Solanum tuberosum cv. Bintje) is synthesized using PCR technology with isolated genomic DNA (Mettler, 1987) as a template. Class-I patatin genes show tuber-specific expression in potato. Both the coding and flanking sequences of several members of the patatin multigene family have been determined (Rocha-Sosa et al., 1989; Bevan et al., 1986; Mignery et al., 1988). Chimeric genes have been reported containing 5' flanking regions of a class-I patatin gene fused to β -glucuronidase, giving rise to tuber-specific expression of β -glucuronidase (Wenzler et al., 1989).

A set of oligonucleotides is synthesized corresponding to the sequence of the pAT21 and B33 genes (Mignery et al., 1989; Bevan et al., 1986), to allow amplification of the class-I patatin 5' flanking region as an HindIII/NcoI fragment:

20 5' ATTAAAGCTTATGTTGCCATATAGAGTAGT 3' and
 5' GTAGGATCCATGGTGCAAATGTTCAAAGTGT 3'.

The oligo's are designed to contain suitable restriction sites (HindIII and NcoI) at their termini to allow assembly of the expression cassette after digestion of the fragments with the restriction enzymes. A fragment of about 1.3 kb, containing a functional class-I patatin promoter fragment is isolated.

The 5' fragment of the class-I patatin gene, as isolated with PCR is cut with HindIII and NcoI. Plasmid pMOG322 (see example 2) contains the mature α -amylase gene from Bacillus licheniformis preceded by an ATG translation initiation codon and followed by the nos terminator from Agrobacterium tumefaciens on a NcoI/HindIII fragment. This fragment is cloned together with the NcoI/HindIII class-I patatin promoter fragment in a three-way ligation into vector pIC19R (Marsh et al., 1984) linearized with HindIII, resulting in vector pMOG435.

The NcoI/HindIII class-I patatin promoter fragment is

cloned together with the BspHI/BglII fragment containing mature glucoamylase from Aspergillus niger preceded by an ATG translation initiation codon (see example 6) in a three-way ligation into vector pIC19R (Marsh et al., 1984) 5 linearized with HindIII and BglII, resulting in plasmid pMOG438. The BamHI/HindIII fragment from plasmid pMOG18 (see example 2), encoding the nos terminator from Agrobacterium tumefaciens is cloned in plasmid pIC19R (Marsh et al., 1984), resulting in plasmid pMOG439. The EcoRI/BglII fragment 10 isolated from pMOG438, containing the class-I patatin promoter and the cDNA encoding mature glucoamylase from Aspergillus niger and the BamHI/EcoRI fragment from pMOG439, containing the nos terminator are cloned in a three-way ligation in pIC19R (Marsh et al., 1984) linearized with 15 EcoRI. The resulting plasmid is designated pMOG440.

B. Cloning of the α -amylase gene from *Bacillus licheniformis* and the glucoamylase gene from *Aspergillus niger* in the 20 binary vector

- Plasmid pMOG435 is digested with HindIII. The HindIII fragment, containing the class-I patatin promoter, the DNA fragment encoding mature α -amylase from Bacillus licheniformis and the nos terminator from Agrobacterium tumefaciens, is cloned in the binary vector pMOG23 linearized 25 with HindIII. This results in the binary vector pMOG436.

Plasmid pMOG440 is digested with EcoRI. The EcoRI fragment, containing the class-I patatin promoter, the cDNA 30 fragment encoding mature glucoamylase from Aspergillus niger and the nos terminator from Agrobacterium tumefaciens, is cloned in the binary plasmid pMOG436, linearized with EcoRI. With restriction enzyme analysis transformants are screened for the presence of the two expression cassettes in a tandem 35 orientation. The binary vector with the expression cassettes having this orientation, called pMOG437 is used for transformation experiments.

The chimeric α -amylase gene from Bacillus licheniformis and the chimeric glucoamylase gene from Aspergillus niger,

both under the control of the tuber-specific class-I patatin promoter, as present on the binary plasmid pMOG437 are mobilized, in a triparental mating with the *E.coli* K-12 strain DH5 α (pRK2013) (Ditta et al., 1980), into 5 *Agrobacterium* strain LBA4404, that contains a plasmid with the virulence genes necessary for T-DNA transfer to the plant (Hoekema et al., 1983).

Example 8.

10 Transformation of potato

Potato (*Solanum tuberosum* cv. Désirée) is transformed with the *Agrobacterium* strain LBA4404 (pMOG437) as described by Hoekema et al. (1989).

15 The basic culture medium is a MS30R3-medium, consisting of MS-medium (Murashige & Skoog, 1962), supplemented with 30 g/L sucrose and with R3-vitamins (Ooms et al., 1987) and where indicated 5 μ M zeatin riboside (ZR) and 0.3- μ M indole acetic acid (IAA). The media are solidified where necessary with 0.7 g/L Daichin agar.

20 Tubers of *Solanum tuberosum* cv. Désirée are peeled and surface-sterilized for 20 minutes in 0.6% hypochlorite solution containing 0.1% Tween-20. The potatoes are washed thoroughly in large volumes of sterile water for at least 2 hours. Discs of approximately 2 mm thickness are sliced from 25 cylinders of tuber tissue prepared with a corkbore. Discs are incubated for 20 minutes in a solution consisting of the MS30R3-medium without ZR and IAA, containing between 10^6 - 10^7 bacteria/ml of *Agrobacterium* LBA4404 (pMOG437). Subsequently, the discs are blotted dry on sterile filter paper and 30 transferred to solid MS30R3-medium with ZR and IAA. Discs are transferred to fresh medium with 100 mg/L cefotaxim and 50 mg/L vancomycin after 2 days. A week later the discs are again transferred to the same medium but this time also with 100 mg/L kanamycin to select for transgenic shoots. After 4-8 weeks shoots emerge from the discs at a frequency of 5-10 shoots per 100 discs. Shoots are excised and placed on rooting medium (MS30R3-medium without ZR and IAA, but with 100 mg/L cefotaxim and 100 mg/L kanamycin), and propagated axenically by meristem cuttings and transferred to soil. The

plants are allowed to tuberize and are subsequently tested for expression of the genes of interest.

5

Example 9

Simultaneous tuber-specific expression of both α -amylase from *Bacillus licheniformis* and glucoamylase from *Aspergillus niger* in potato and sugar and starch analysis of transgenic tubers

Transgenic plants are assayed for both α -amylase and
10 glucoamylase activity. Alfa-amylase activity is determined as described in Example 4. Glucoamylase activity is determined using the peroxidase-glucose oxidase (PGO)-o-dianisidine (ODAD)-assay (Sigma kit #510) which detects glucose released from soluble starch by the glucoamylase. One glucoamylose
15 unit is defined as the amount of glucoamylose which releases one μ mol of glucose per minute from washed soluble starch at 37 °C. Plant material (about 50 mg) is homogenized in 100 μ l assay buffer and homogenized. The homogenate is spun for 10 minutes in an Eppendorf centrifuge. The supernatant is tested
20 for both α -amylase and glucoamylase activity and for protein content. Activity is only found in the tubers of the transgenic potatoes. The proteins of interest are also detected with antibodies raised against the purified proteins on Western blots.
25 Tubers of transgenic potatoes expressing both enzymes are analyzed for the presence of soluble and insoluble saccharides by HPLC and NMR, respectively. A higher content of soluble sugars is found in transgenic tubers as compared to control plants.

30

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CLAIMS

1. A method for modifying the carbohydrate composition of a plant or plant organ characterized by the growing of a transgenic plant containing an expression cassette which contains a DNA sequence encoding an enzyme of interest "capable" of degrading a plant polysaccharide, under conditions conducive whereby said enzyme-encoding DNA sequence is expressed and the polysaccharide composition of said plant or plant organ is modified.
2. The method of claim 1 further characterized in that said expression cassette contains a regulatory sequence capable of directing the expression of said enzyme of interest at a selected maturity stage of the development of the transgenic plant or plant organ.
3. The method of either of claims 1 and 2 further characterized in that said expression cassette is capable of directing the tissue-specific expression of said enzyme of interest.
4. The method of claim 3 further characterized in that an increase in the content of soluble saccharides containing up to six monosaccharide units is obtained in said transgenic plant or plant organ as a result of the action of said enzyme of interest.
5. The method of any one of claims 1 - 4 wherein said enzyme of interest is an amylase.
6. The method of any one of claims 1 - 5 further characterized in that said transgenic plant contains one or more expression cassettes containing DNA cassettes encoding an enzyme of interest other than and in addition to said first enzyme capable of using polysaccharides as a substrate, said additional enzyme of interest being capable of using the starch degradation products resulting from the action of said first enzyme of interest as a substrate.
7. The method of claim 6 further characterized in that said

additional enzyme of interest is selected from the group consisting of starch degrading enzymes, starch debranching enzymes, cellulases, endo- β -1,3/1,4- β -glucanases, L-arabinases, galactanases, mannanases, xylanases, α -L-fucosidase, α -L-rhamnosidase, levanase and inulinase.

8. The method of any one of claims 1 - 7 further characterized in that said transgenic plant is a plant which is used for consumption, either as such or in further processing, by domesticated animals or humans.

9. A transgenic plant, obtained through application of a process according to any of the above claims, as well as cells and parts of said plants and their progeny obtained through sexual or asexual propagation.

10. An expression construct characterized in that a DNA sequence encoding an enzyme of interest capable of degrading a plant polysaccharide is operably linked to a regulatory sequence capable of directing the expression of said enzyme of interest at a selected maturity stage of the development of a transgenic plant or plant organ.

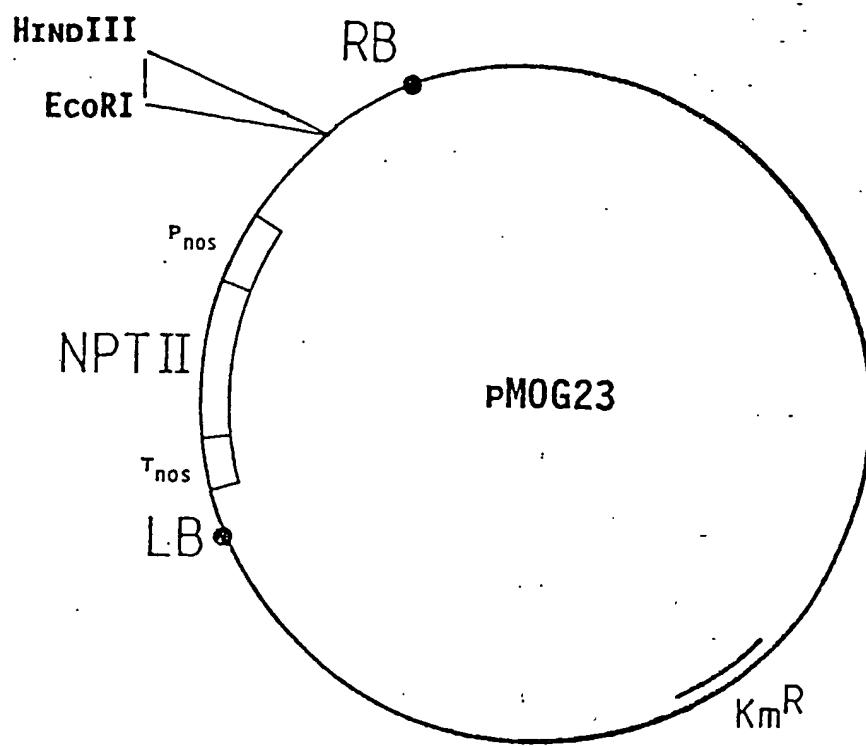
11. An expression construct characterized in that a DNA sequence encoding an enzyme of interest capable of degrading a plant polysaccharide is operably linked to a regulatory sequence capable of directing the tissue-specific expression of said enzyme of interest.

12. A vector comprising an expression construct according to any of the claims 10 or 11.

13. Bacterial strains containing a vector according to claim 12.

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8



Polylinker sequence:

Eco RI	Kpn I	Sma I	Bam HI	Xba I	Xho I	Hind III
5' GGAATTCTGGTACCTCCCAGGATCCATCTAGAGCTCGAGTAAGCTTC 3'						
					Sac I	

Fig. 1

25

XBAI

TCTAGAC TC ATGAAACACAAAAACCGCTTTACCCCCCATTCCTCACCCCTGTATTTCGGCTCATCTTC
MetLysGlnGlnLysArgLeuTyrlsAeqLeuLeuLeuLeuLeuPheAlaLeuIlePhe -10

PSTIHGAII

TTGCTGCCCTCATCTGCACCCGGGGGGCAAACTTTAATGCCACCTGATGCCACTATTTCAATGGTACATGCCCAATCAGCCCAACATTGGAAAGCCCTTCCAAAACGACTCCCCATAT
LeuLeuProHisSerAlaAlaAlaAlaAsnLeuAsnGlyThrLeuMetGlnTyrcleGluTrpTyrcleProAsnAspGlyGlnHistpLysAeqGluGlnAsnAspSerAlaIleTyr
-1 8 +1 31

TTGCCCTGAAACACCGTATTACTGCCGCTGCGATTCGGGGCATATAAGCCAAACGCCAACGGCCATGTCGGCTACGGCTCCATGCCAACTTACGGGAGCTTCATCAAACAAACGGC
LeuAlaGluHisGlyIleThrAlaValTrpIleProProAlaTyrlsGlyThrSerGlnAlaAspValGlyTyrGlyAlaIleTyraSpLeuGlyGluPheHisGlnLysGly
71

ACGGTTCCGACAAACTACGCCAACAAAGGAGAGCTGCAATCTGGCATCAAAGCTCTCATTTCCCCCAACATTAAAGCTTACCCGGATGTGTCATCAACCACAAAGGGGGCTGATGCC
TheValIargThrLysTyrlsGlyGluLeuGlnSerAlaIleLysSerArgAspIleAsnValTyrlsGlyAspValValIleAsnHisIleGlyGlyAlaAspAla
111

ACCCAAAGATGTAACCCCCGGTGAAGTCGATCCCGCTGACCCGAAACCCGTAATTTCAGGAAACACCTAATTAAAGCTGGCACACATTTCATTTCAACAAAGGGCTTCCGAACTTCCAAATGAAACGGC
TheGluAspValThrAlaValGluValAspProAlaAspArgAsnArgValIleSerGlyGluHisLeuIleLysAlaTrpThrHisPheHisPheProGlyArgGlySerThrTyrcle
151

GATTTAAATGCCATTTGGTACCCATTTGACCCGATGGGACCCAGCTCCCAAAAGCTGACCCGAACTTAACTTCAAGGAAAGGGCTTCCGAACTTCCAAATGAAACGGC
AspPheLysTrpHisTrpTyrclePheAspGlyThrAspTrpAspGluSerArgLysLeuAsnArgIleTyrlsGlyPheGlnGlyLysAlaIleTrpAspTrpGluValIleAsnGluAsnGly
191

AACATATGATTATTCGATGATCTCCGACATCGGATTATGACCATCTCTGATGTCGCAACGAAATTAAAGACATGGGCAACTTGGCTATGCCAAATGAACTCCAAATTGGACGGTTTCCGCTTGT
AsnTyrcleAspTyrlsLeuMetTyrlsAlaAspIleAspTyrlsProAspValIleAlaGluIleLysArgTrpGlyThrTrpTyrlsAlaAsnGluIleLeuGlnLeuAspGlyPheArgLeuAsp
231

GCTGTCAAACACATTAATTTCCTTTGGGCAATTGGGTTAACATGTCAGGAAACGGGAAAGGAAATGTTACGGTAGCTGAATATTGGCAGAAATGACTTGGGGCCCTGCCAA
AlaValIleLysHisIleLysPheSerPheLeuArgAspTrpValAsnHisValIleArgGluLysTheGlyLysGluMetPheTheValAlaIleGluTyrlsTrpGlnAsnAspLeuGlyAlaIleGlu
271

AACTATTCGACAAACAAATTAAATCATTCAGTGTTCACCTGCCGCTTCATTTGACTTCCATGCCATGACACAGGGAGCCCCTATGATATGAGGAAATTGCTGAGGGTACCG
AsnTyrlsLeuAsnLysTheAsnPheAsnHisIleSerValIleAspValPheAspValProLeuHisAlaAlaSerThrGlnGlyGlyTyrAspMetArgLysLeuLeuAsnGlyThr
311

CTCAXAAGGAATCTGGATACCCCTCAGCTTTCACGGGATATGACGGGACGAAAGGAGACTCCCAGCGCAAAATTCCTGGCTGAAACACAAACATTGAAACGGATCTTAAAGGCA
LeuThrArgGluSerGlyTyrProGlnValPheTyrlsGlyAspMetTyrlsGlyTrpLysGlyAspSerGlnArgGluIleProAlaIleLeuLysHisIleGluProIleLeuLysAlaIleArg
391

AAACAGTATGGCTACGGAGCACAGCATGATTTCGACCAACCATGACATTGCTGGGACAAGGGAGGGCACACCTGGCTGGCAATTTCAGCTTGGGGCCATTAAATAACAGACCCA
LysGlnTyrlsAlaGlnHisIleAspTyrlsPheAspHisIleAspIleValGlyTrpThrArgGluGlyAspSerSerValAlaAsnSerGlyLauAlaAlaIleLeuIleThrAspGly
431

CCCCGTGGGGCAAGGCAATGATGTCGGGGGGCAAAACCCGGCTGAGACATGCCATCACATTACCCAAACCCCTTCCGAGCCGGTTCTCATCAATTGGAAAGGGCTGCCAGACACTTCAC
ProGlyGlyAlaLysArgMetTyrlsValGlyArgGlnAsnAlaGlyGluThrTrpHisAspIleTheGlyAsnArgSerGluProValValIleAsnSerGluGlyTrpGlyGlyPheHis
471

GTAAACGGGGGGTGGTTCAATTATGTTCAAGGATAGAGACGACAGAGGACCGGATTTCCTGAGGAAATCCGTTTTTATTGCCCCCTCTATAAATTCTTCTTCAATTAC
ValAsnGlyGlySerValSerIleTyrlsValGlnArg***
483

TAATTAATTAAACAAACTGTCATCAGCCCTCAGGAACCACTTGTGAGCTTGTGATGCCATACGTAACGGGGATGAAATGCCAACGTTATGATGTCACAAAGAACCAAATGTC

BCL1

TCCAAAATGACGGTATCCGGGTGATCA

Fig 2

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Figure 3.

Oligonucleotide duplex A.

5

Oligonucleotide duplex A

10

NcoI BamHI HindIII

5' GGGTTTTATTTTAATTTCTTCAAATACTTCCACCATGGTAACGGATCCA 3'
3' CCCAAAAATAAAAATTAAAGAAAAGTTATGAAGGTGGTACCCATTGCCCTAGGTTCGA 5'

15

Oligonucleotide duplex B

20

NcoI HpaI SITE
| || | |
| | | |

5' CATG|GCAAATCTTAATGGACCGCTGATG 3'
3' |CGTTTAGAATTACCTGCGACTACCTCAT 5'

25

Mature α -Amylase

|

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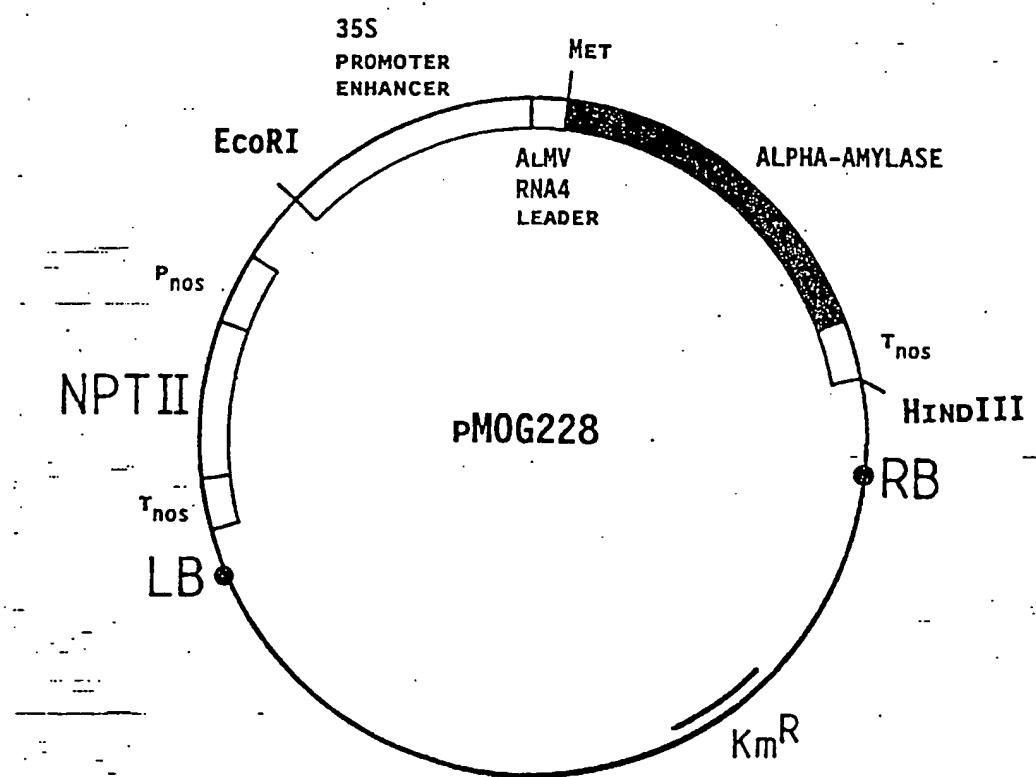


Fig. 4

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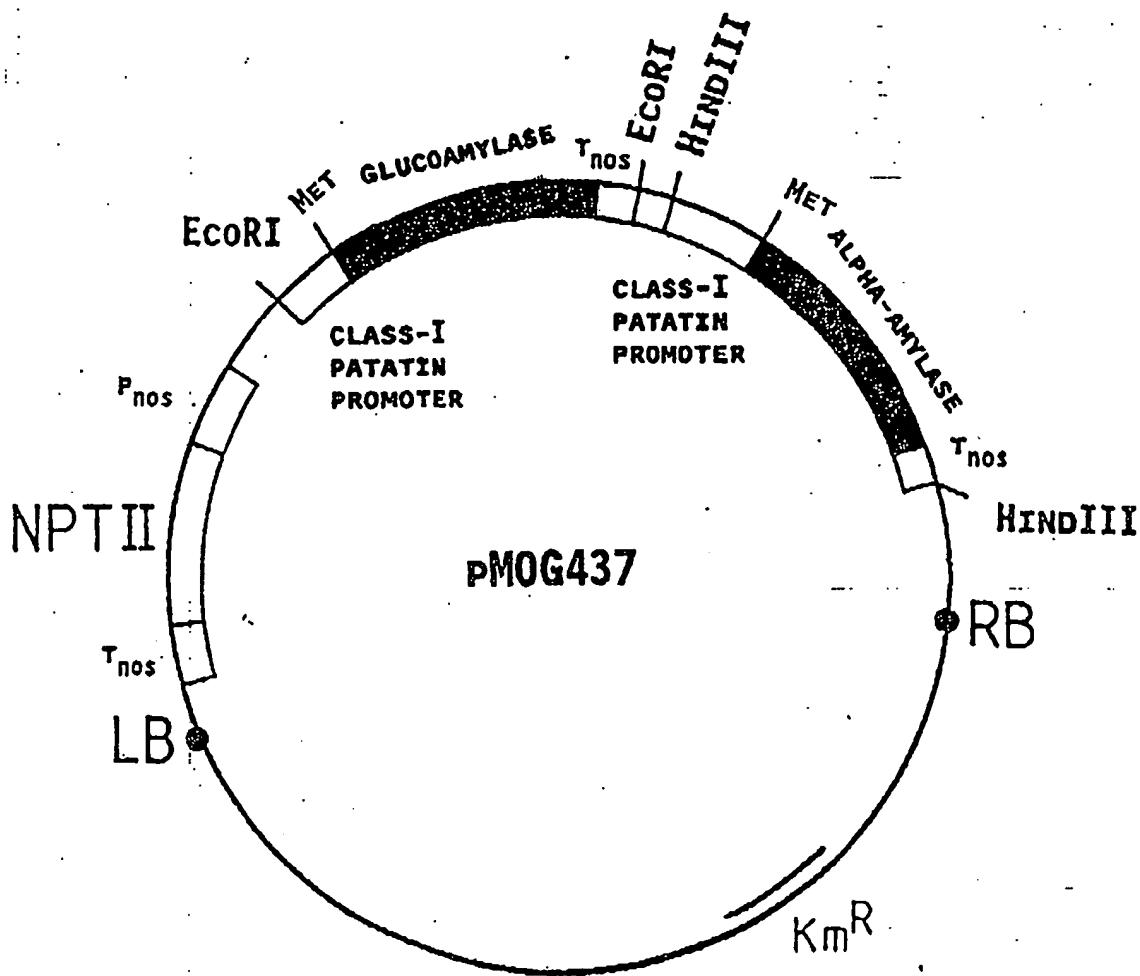


Fig. 5

ABSTRACT

The present invention provides plants with a modified taste and/or texture. The invention also provides methods to obtain such plants, which by means of known techniques have been regenerated from plant cells or other plant parts, transformed with DNA constructs containing genes encoding enzymes capable of degrading plant polysaccharides and eventually, in addition, genes encoding enzymes that are capable of modifying the degradation products resulting from the first degradation step. The invention is explicitly exemplified with tobacco plants expressing an α -amylase gene from Bacillus licheniformis under the regulation of the CaMV 35S promoter and with potato plants expressing both the α -amylase gene mentioned above and a glucoamylase gene from Aspergillus niger, both under the control of the tuber-specific class-I patatin promoter from potato.